STUDIES ON THE MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX: PYRUVATE DEHYDROGENASE KINASE BINDING SITES, REDUCTIVE ACETYLATION REACTION AND EXTRINSIC CONTROL

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TO MY GRANDMOTHER

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LIST OF ABBREVIATIONS

DTT	Dithiothreitol
E1, PDH	Pyruvate dehydrogenase component
E2	Dihydrolipoyl transacetylase component
$E2^*$	Dihydrolipoyl transacetylase with low level of protein X
$E2_I$	The inner domain of dihydrolipoyl transacetylase component
$E2_L$	The outer (lipoyl-bearing) domain of dihydrolipoyl dehydrogenase component
$E2-X_I$	Dihydrolipoyl transacetylase-protein X inner domain subcomplex
$E2-X-K_cK_b$	Dihydrolipoyl transacetylase-protein X-kinase subcomplex
E3	Dihydrolipoyl dehydrogenase component
FAD	Flavine-adenine dinucleotide
HETPP	Hydroxyethyl thiamin pyrophosphate
IP	myo-inositol 2-monophosphate
IPG	Inositol phosphate glycan
K_c	The catalytic subunit of pyruvate dehydrogenase kinase
K_b	The basic (high pI) subunit of pyruvate dehydrogenase kinase
Kinase	Pyruvate dehydrogenase kinase
NAD (NADH)	Nicotinamide-adenine dinucleotide
PDC	Pyruvate dehydrogenase complex
Phosphatase	Pyruvate dehydrogenase phosphatase
PIPLC	Phosphatidylinositol-specific phospholipase C
TPP	Thiamin pyrophosphate
X_I	The inner domain of protein X
$X-K_cK_b$	Protein X-kinase subcomplex
X_L	The outer (lipoyl-bearing) domain of protein X

Chapter 1

Pyruvate Dehydrogenase Kinase Binding Study

INTRODUCTION

The pyruvate dehydrogenase complex (PDC) performs a central function in carbohydrate oxidation and fatty acid synthesis. In the presence of thiamin pyrophosphate (TPP) and Mg^{2+}, it catalyzes the conversion of pyruvate to acetyl-CoA. This is a key step in the oxidation of carbohydrate and is a rate limiting step in the biosynthesis of fatty acid. This reaction involves the coordinated functioning of three catalytic components: pyruvate dehydrogenase (PDH or E1), dihydrolipoyl transacetylase (E2) and dihydrolipoyl dehydrogenase (E3). The individual steps catalyzed by these components are given in the introduction to chapter 2. The overall reaction can be represented by:

$$\text{Pyruvate} + \text{CoA} + NAD^+ \rightleftharpoons \text{Acetyl-CoA} + CO_2 + \text{NADH} + H^+$$

The pyruvate dehydrogenase component of PDC is regulated by interconversion between an active, nonphosphorylated form and an inactive, phosphorylated form (1) by two regulatory enzymes, PDH kinase and PDH phosphatase that are associated with PDC (9).

The inner core of PDC is thought to be composed of 60 identical dihydrolipoyl transacetylase (E2) subunits (19). Each subunit has two domains (1). The inner domain ($E2_I$) of E2 associates to form the large oligomeric core and catalyzes the transacetylation reaction (1). The outer domain ($E2_L$) is the lipoyl-bearing region that participates in the transfer of the

acetyl group. Another protein referred to as protein X (3,4,7,14,16) is tightly bound to the E2 core (14). Protein X is structurally distinct from E2 subunit, and also has two domains: an inner domain (X_I) which binds to E2 core, and a lipoyl-containing outer domain (X_L) that undergoes reduction and acetylation (17). Protein X lacks the transacetylation activity of E2 subunits (15).

The pyruvate dehydrogenase kinase consists of a catalytic subunit (K_c) and a basic subunit (K_b) (14,21). While the role of K_b has not been well established, recent studies have shown that an increase in kinase activity is correlated with the cleavage of the K_b subunit, suggesting that K_b might inhibit the activity of K_c (17). Approximately three kinase molecules are bound to each E2 core (7,14). The protein X and kinase subunits are both tightly bound to E2 and are retained after the resolution process that removes the E1 and E3 components (7,10). It also has been demonstrated that the reduction and acetylation of protein X prepared free of E2 subunits is associated with a small enhancement of kinase activity (15). It was proposed that protein X plays a role in attenuating kinase activity (15) and serves as an anchor of kinase to the E2 core (14). However, a recent study involving protease Arg C digestion of E2-X-K_cK_b revealed that removal the outer domain from E2 subunit released K_c subunits, thus providing evidence that the kinase is anchored on E2 directly (17). In addition there is evidence that E3 subunits directly associate with protein X (18).

Since it is not clear how protein X or kinase are bound to the E2 core, it is interesting to characterize their ability (following their removal) to reassociate with the E2 core containing or lacking protein X and kinase molecules.

EXPERIMENTAL PROCEDURES

Materials: Highly purified bovine kidney pyruvate dehydrogenase complex (15-18 μmole/mg/min) was resolved to E2-X-K_cK_b subcomplex by the procedure of Linn *et al.* (10), X-K_cK_b fraction by the procedure of Stepp *et al.* (21), E2-X_I was generated by protease Arg C treatment (17). E2* (E2 with low level of protein X) was resolved by urea and NaCl by the procedure of Powers-Greenwood and Roche (unpublished procedure). Fig 1-1 diagrams these different preparations. [γ-^{32}P]ATP was from ICN Biomedicals, Inc. [^{14}C]ATP and [1-^{14}C]acetyl-CoA were purchased from New England Nuclear (NEN) Du Pont Company. Bovine serum albumin (BSA) as protein standard was from U.S. Biochemical Corporation. All other chemicals used were highest grade available.

Kinase binding experiment: E2 component and kinase sources were incubated at desired levels at room temperature in buffer which contained 50 mM Na_xPO_4, pH 7.5, 0.2 mM ethylenediamine tetraacetic acid (EDTA) and 0.5 mM dithiothreitol (DTT) in a total volume of 50 μl. After 15 min, the samples were overlaid on a sucrose gradient in airfuge tubes. Sucrose step-gradients (7.5%, 10%, 15% w/v) were preformed in the same buffer as mentioned above. The volume of the each step was 20 μl. Samples were centrifuged in SW 29 rotor at 4°C, 151200xg for 90-120 min. After centrifugation, supernatants were withdrawn and one half volume (55 μl) of the same buffer was added to dissolve the pellet for 14

h on ice.

O-phthaldehyde protein measurement: The protein concentration of each supernatant and pellet fraction was determined by the procedure of Fried *et al.* (5). Excitation and emission wavelengths were 330 nm and 418 nm respectively. BSA was used as protein standard.

Kinase activity assay: Pyruvate dehydrogenase kinase activity was determined as the initial rate of incorporation of ^{32}P-phosphoryl groups into protein from [γ-^{32}P]ATP (13). Assays were conducted at 30°C in the buffer which contained 20 mM K_xPO_4, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EDTA and 2 mM DTT. The order of addition of the various components was as follows: E1, E2-X subcomplex and kinase source. The mixture was preincubated for 1-2 min and [γ-^{32}P]ATP ($1-3x10^5$ cpm/nmole) was added to a final concentration of 100 µM to start the reaction. The total volume was 50 µl. At designated time intervals, 20 µl was withdrawn and applied on a dry disc filter paper presoaked with 10% trichloroacetic acid (TCA) to stop the reaction. Three 30-min washes and another 14-h wash with 10% TCA were done, then followed by two 5-min absolute ethanol washes and two 2-min ethyl ether washes. Assays were done in duplicates and average values were reported.

Transacetylation activity assay: The assays were conducted as described by Butterworth *et al.* (2). Dihydrolipoyl transacetylase

catalyzes the transfer of the acetyl group of [1-^{14}C]acetyl-CoA to dihydrolipoamide (DHL). The radioactive S-acetyl dihydrolipoamide is extracted into benzene. The reaction mixture contained 25 mM K_xPO_4, pH 7.5, 0.5 mM DHL, 0.05 mM EDTA and 0.5 mM [1-^{14}C]acetyl-CoA ($6x10^5$ cpm/μmole) to make a total volume of 0.5 ml. The reaction was started by the addition of [1-^{14}C]acetyl-CoA and the mixture was incubated at room temperature for 2 min. 1 ml benzene was added to stop the reaction. The mixture was shaken for 10 s on a vortex to extract the S-acetyl DHL. A 0.2 ml aliquot of benzene layer was withdrawn and radioactivity was measured by the scintillation counting. All assays were done in duplicate.

ATP binding experiment: Binding of [^{14}C]ATP (97,000 cpm/nmole) to the PDH kinase was measured by the procedure of Pratt *et al.* (12). Millipore filters were presoaked with 5 mM ATP in 20 mM K_xPO_4, pH 7.0 to reduce nonspecific binding, then washed with 5 ml of cold 20 mM K_xPO_4, pH 7.0. The assays were done in buffer containing 50 mM MOPS-K^+ (3-(N-morpholine)propane sulfonic acid) pH 7.3, 60 mM KCl, 2 mM DTT, 1.5 mM $MgCl_2$ and 0.5 mM EDTA. The kinase-containing fractions were added and the reactions were started by the addition of [^{14}C]ATP to the final concentration of 180 μM. The mixture was incubated at 22°C for 90 s in a total volume of 25 μl. Then a 20 μl aliquot was withdrawn and applied to the cold membrane filter, and immediately washed six times with 2 ml of cold 20 mM K_xPO_4 buffer. After the membrane dried, the radioactivity was measured. All assays were done in duplicate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): SDS slab gel electrophoresis was performed under the conditions described by Laemmli (8) with 10% acrylamide in the running gel and 5% in the stacking gel. Bands were visualized with a silver stain following the procedure by Oakley *et al.* (11).

RESULTS

Analysis of the binding of the kinase and protein X subunits to the E2 core.

The binding of kinase to E2 subunits was studied with two ratios of the X-K_cK_b fraction to E2-X-K_cK_b subcomplex. Figure 1-2 shows the gel pattern when the lower ratio of X-K_cK_b fraction to E2-X-K_cK_b subcomplex was used. By comparing protein bands of the pellet fraction (figure 1-2, X-K_cK_b/E2-X-K_cK_b: P1, P2) with the standard E2-X-K_cK_b (figure 1-2, first six lanes), P1 had about the same amount of E2 component as E2-X-K_cK_b standard in lane 3 (0.4 µg), also E2 band in P2 (half of the sample of P1 loaded) matched well the E2 band in lane 5 of E2-X-K_cK_b standard (0.2 µg). The K_c bands of the P1 and P2 pellets showed a large increase in the level of K_c relative to the original E2-X-K_cK_b subcomplex. Even when the P2 was compared to the highest level of the E2-X-K_cK_b (which had three times E2), there was a higher level of K_c present.

The supernatant fraction (figure 1-2, X-K_cK_b/E2-X-K_cK_b: S) had some residual kinase which indicated that it was not as tightly associated with the E2 core. It was uncertain whether this reflected saturation of kinase binding sites, the binding equilibrium, or some damage to a portion of the kinase molecules during preparation. In order to determine whether the E2 binding with kinase had reached the maximum capacity, a higher ratio of X-K_cK_b fraction/E2-X-K_cK_b subcomplex was incubated, centrifuged and analyzed by SDS-PAGE (figure 1-3). The same pattern of E2-X-K_cK_b

subcomplex standards was used (figure 1-3, first six lanes). The gel pattern showed even more kinase (K_c) subunits binding to the E2-X-K_cK_b subcomplex (fig 1-3, X-K_cK_b/E2-X-K_cK_b: P1, P2, P3). Again a significant increase in protein X binding was not detected. More kinase remained in the supernatant (Figure 1-3, X-K_cK_b/E2-X-K_cK_b: S), presumably due to an excess of added kinase, although the alternative explanation mentioned above may still be relevant.

While additional kinase was bound to E2-X-K_cK_b subcomplex, there was little or no increase in the amount of protein X associated with the E2-X-K_cK_b subcomplex. This brought up the question concerning the nature of the association of protein X with the E2 core. It was found in the above experiments that the amount of protein X associated with E2 core did not significantly increase along with the increased binding of K_c, but it is not clear whether the protein X subunit in the X-K_cK_b fraction can undergo exchange with those on the E2 core or whether protein X occupies sites not available for reversible protein X binding.

In an attempt to clarify these questions, another binding experiment was conducted with the E2-X_I subcomplex in which the outer domain of protein X was removed but the inner domain still occupied the protein X sites on the E2 core and the $E2^*$ which is a form of the E2 oligomer with most of protein X removed. The results are shown in figure 1-4. Again increased K_c binding was observed. However little or no protein X present in X-K_cK_b fraction was bound to $E2^*$ and E2-X_I subcomplex (Figure 1-4, X-

$K_cK_b/E2-X_I$: P1, P2, P3; $X-K_cK_b/E2^*$: P1, P2, P3). These results suggested that the protein X in the $X-K_cK_b$ fraction did not exchange with the inner domain of protein X (X_I) that occupied the protein X binding sites; protein X did not bind to available sites on $E2^*$ either. However the possibility that protein X was damaged during the resolution steps and therefore lost the ability of binding to E2 core can not be eliminated.

PDH kinase binding sites on the E2 core.

In order to determine the number of PDH kinase molecules bound per E2 core and to evaluate whether the unbound kinase in the supernatant was still native, kinase ATP binding and specific activity studies were conducted.

The ATP binding capacity of the kinase was determined for all the kinase containing fractions. The initial content of K_c in the $E2-X-K_cK_b$ subcomplex and the amount of $X-K_cK_b$ fraction added set the lower and the upper limits for the amount of kinase that could be bound. Measurement of the ATP binding of the pellet fractions from the first and the second experiments confirmed increased capacity for binding of ATP (3 and 5.5 fold respectively) (Table 1-I). The kinase remaining in the supernatants also exhibited the ability to bind the ATP.

Although kinase was still capable of binding ATP, it is important to examine its catalytic function to see whether it is native. The kinase activity of each fraction was measured. To attempt to assure full expression of kinase activity, E2-X

subcomplex was added in some assays. The capacity of the E2-X subcomplex to increase kinase activity is recognized from the difference between the activities of the samples assayed with and without E2-X subcomplex (table I-1). Also the activation of the large number of K_c associated with the E2 core present in the pellet fractions is evaluated without extra E2-X subcomplex in the assay.

When measuring the specific activity, it is important to determine the initial velocity. Since a large number of kinase molecules are present in the pellet fractions, substrate (pyruvate dehydrogenase component) depletion is a potential problem. Thus it was necessary to keep the reaction time as short as possible so that there would be only a few turnovers before the substrate depletion occurs. After a few turnovers, the exchange between the free active pyruvate dehydrogenase and bound inactive pyruvate dehydrogenase on the E2 core could be the rate limiting factor.

Actually, in the pellet fractions, large kinase activations by E2-X subcomplex were not observed (table 1-I., numbers in the parenthesis). This is informative since pellet fractions contained E2-X subcomplex. The calculations of specific activity of kinase per ATP binding were made from ATP binding and kinase specific activity study of the two pellet fractions, the numbers ranged from 30-40. This is similar to the range (27-34) reported for the kinase associated with PDC complex, the E2-X-K_cK_b subcomplex and the X-K_cK_b fraction in another study (14). This suggested that the kinase in the pellet fractions maintained full

activity, also the E1 associated with the E2 core were being made available to the large number of kinase molecules.

However, the kinase in the supernatant of lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex was not activated. Kinase in the supernatant of higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex was activated a little, but not as much as expected. Since K_c did not exhibit activation by E2-X, it is possible that free kinase lost a portion of its catalytic capacity. Earlier experiment did show that kinase in the supernatant fractions had the ability to bind ATP. A factor that may have contributed to the low kinase activity and its lack of activation was that the kinase was stored frozen in a small aliquot for more than two weeks prior to the kinase activity assays. It is not certain whether this activity loss occured during that storage or reflected a defective portion of the K_c in the initial X-K_cK_b fraction. However it is known that the kinase in the X-K_cK_b fraction is less stable to freezing and thawing than the kinase associated with the E2-X subcomplex (unpublished observation).

Based on the kinase ATP binding and E2 activity studies, calculations were made to estimate the maximum numbers of kinase bound to E2 (Table 1-II). The binding number determined from the pellets in which the lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex experiment was about 9 kinase molecules per E2 core. Based on the amount of X-K_cK_b fraction and E2-X-K_cK_b subcomplex used, the maximal possible binding was about 10. Since essentially all the kinase available was bound, it was not certain if the outcome

reflected saturation of the potential binding sites for kinase. The experiment was repeated using the higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex attempting to increase the K_c binding to E2. The maximum possible kinase binding was 27. This time a number of about 16 kinase molecules bound to each E2 core was derived.

DISCUSSION

It is known that protein X and PDH kinase associate tightly with the E2 core to form E2-X-K_cK_b subcomplex. However how these components are organized is not well known. There are about 6 protein X components bound to the E2 core (7). It was proposed that protein X serves as the anchor through which the PDH kinase is attached to E2 core (14). This was based on the result of previous studies of the activation of the kinase. The experiments were performed with E2-X subcomplex and X-K_cK_b fraction. A fixed level of E2-X subcomplex and pyruvate dehydrogenase component was titrated with kinase. The data indicated that the E2-X subcomplex had a high capacity for activation of the kinase in the X-K_cK_b fraction. Since kinase must be bound to the E2 core to be activated, the increased kinase activity reflected the increase in kinase binding.

Whether this increased kinase activity was due to the extra protein X binding which may serve as an anchor for kinase or was due to the direct kinase binding to the E2 core was not clear. Also an earlier binding experiment done with E2-X subcomplex and X-K_cK_b fraction provided evidence that along with increased binding of kinase, that there was an increase in the binding of protein X to the E2 core in the pellet fraction (unpublished observation). This observation was consistent with the suggestion that protein X played an anchor role in the kinase binding to the E2 core.

To further study the nature of protein X and kinase binding

to the E2 core, binding experiments were conducted using E2-X-K_cK_b subcomplex, E2-X_I subcomplex, E2* and X-K_cK_b fraction. Great care was invoked in the preparation of the X-K_cK_b fraction to thoroughly remove the mercurial agent in its preparation. From the SDS gel patterns of E2-X-K_cK_b subcomplex binding with X-K_cK_b fraction (figure 1-2, 1-3), it is obvious that the amount of kinase bound to the E2 core increased significantly. However, an increased protein X level was not observed. This suggested that kinase associated with E2 directly instead of through protein X. It is also possible that each protein X component has the ability to bind more kinase molecules. But this possibility was eliminated by the binding experiments done with two other fractions. E2-X_I subcomplex and E2* exhibited the same ability to bind kinase (figure 1-4). These results provided the evidence that kinase was bound to E2 core directly. Since previous data showed that kinase did not bind to the inner domain of E2 ($E2_I$) (14), it might anchor to the lipoyl-bearing domain ($E2_L$). Another study found that removal of outer domains from E2 subunit released the K_c subunits of kinase (17).

The experiments done above strongly suggested that protein X was not involved in the kinase binding to E2 core. How protein X binds to E2 core is not well understood. Although the structure of protein X is distinct from the E2 subunit, the peptide mapping, sequencing and immunological studies revealed that they have structurally distinct but related lipoyl domains (7,18). The kinase binding experiments done with E2-X_I subcomplex and E2*

provided new insights into the possible nature of that protein X binding. It is evident that the protein X in the $X\text{-}K_cK_b$ fraction neither underwent exchange with the inner domain of protein X (X_I) associated with E2 core nor occupied the available protein X binding sites in $E2^*$ fraction from the SDS gel analysis. It seems reasonable to speculate that instead of being associated with the periphery of the E2 core, protein X component may be integrated into E2 subunits as part of the E2 core. If this is the case, it would be of interest to investigate how the E2 core with protein X is assembled and whether there is a fixed number of protein X molecules assembled into each E2 core. Another less intriguing possibility is that protein X in the $X\text{-}K_cK_b$ fraction was denatured by the treatment of organic mercurial reagent during the resolution steps, therefore losing its binding ability. But the same $X\text{-}K_cK_b$ fraction was shown to bind the E3 component (unpublished results), which suggests that protein X has preserved a major portion of its native structure. Although protein X probably does not participate in kinase binding, reduction and acetylation of its lipoyl domain in the absence of the E2 subunits was associated with a small activation of the kinase (15). The latter study was also performed with the $X\text{-}K_cK_b$ fraction and further indicates that the protein X in the fraction maintained some native structure (18).

The control fractions ($E2\text{-}X\text{-}K_cK_b$ subcomplex, $E2\text{-}X_I$ subcomplex and $E2^*$ centrifuged alone) did not pellet as well as the fractions containing $X\text{-}K_cK_b$. Although the binding of kinase to the E2 core

must increase the molecular weight of the subcomplex, this increase would be expected only to be from $3.6x10^6$ to about $4.4x10^6$ which could not account for the observed increase in the sedimentation.

Combining the results from kinase activity assay, kinase ATP binding and E2 transacetylation activity assay, a maximum of 16 kinase molecules was bound to each E2 core. In both lower $X-K_cK_b$ fraction/E2-X-K_cK_b subcomplex and higher $X-K_cK_b$ fraction/E2-X-K_cK_b subcomplex experiments, there was a trace and moderate amount of kinase remaining in the supernatant fractions. It was necessary to examine whether these kinase still possessed the native properties. The kinase ATP binding study revealed that it preserved ability to bind ATP and the amount of ATP bound was proportional to the kinase present in each supernatant fractions. But when its catalytic function was examined, little kinase activation was observed. This indicated that kinase in the supernatant had lost its ability to be activated. It is not clear what caused this loss in activation capacity. Perhaps the kinase in the supernatant fractions was denatured during storage, which included freezing and thawing. In contrast, the kinase in the pellet fractions maintained a high activity. It is possible that kinase may be stabilized and maintain its high activity at the presence of E2 subunits.

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Figure 1-1. Scheme of the stages in the purification and resolution steps in the preparation of pyruvate dehydrogenase complex and resolved components.

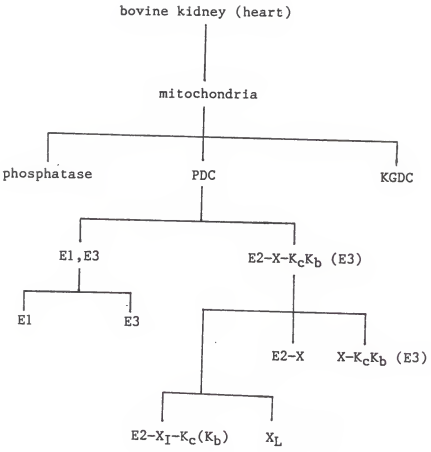
bovine kidney (heart)
mitochondria
phosphatase
PDC
KGDC
E1,E3
E2-X-KcKb (E3)
E1
E3
E2-X
X-KcKb (E3)
E2-XI-Kc(Kb)
XL

Figure 1-2. SDS-PAGE of PDH kinase binding using lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex. X-K_cK_b fraction (40 µg) and E2-X-K_cK_b subcomplex (60 µg) were incubated at room temperature for 15 min, then loaded on sucrose gradient and centrifuged. The samples were analyzed by SDS-PAGE and visualized by silver gel staining (refer to experimental procedures for details). First six lanes from left are E2-X-K_cK_b subcomplex standards, protein levels loaded decreasing from 0.6 µg to 0.1 µg; **X-K_cK_b/E2-X-K_cK_b: S** is the supernatant fraction (0.34 µg) of lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex; **X-K_cK_b/E2-X-K_cK_b: P1, P2** are pellet fraction of lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex, the two protein levels (0.4 µg, 0.2 µg) were loaded in order to precisely compare with E2-X-K_cK_b subcomplex standards; **E2-X-K_cK_b: S** is the supernatant fraction (0.12 µg) of control E2-X-K_cK_b subcomplex; **E2-X-K_cK_b: P** is the pellet fraction (0.56 µg) of control E2-X-K_cK_b subcomplex; **X-K_cK_b: S** is the supernatant fraction (0.44 µg) of control X-K_cK_b fraction; **X-K_cK_b: P** is the pellet fraction (0.02 µg) of control X-K_cK_b fraction; **X-K_cK_b** is X-K_cK_b fraction, two levels were loaded (1.0 µg, 0.5 µg); **PDC** is pyruvate dehydrogenase complex (1.0 µg).

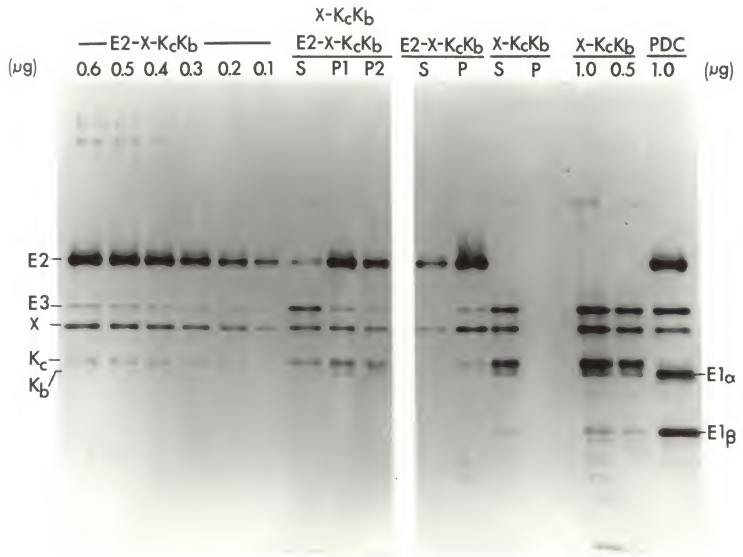
X-KcKb
E2-X-KcKb
E2-X-KcKb
E2-X-KcKb
X-KcKb
X-KcKb
PDC
(μg)
0.6 0.5 0.4 0.3 0.2 0.1
S P1 P2
S P
S P
1.0 0.5
1.0
(μg)
E2
E3
X
Kc
Kb
E1α
E1β

Figure 1-3. SDS-PAGE of PDH kinase binding using higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex. X-K_cK_b fraction (60 µg) and E2-X-K_cK_b subcomplex (30 µg) were used in the binding experiment as mentioned in the legend of figure 1-2. First six lanes from left are E2-X-K_cK_b subcomplex standards (0.6 µg-0.1 µg); **X-K_cK_b/E2-X-K_cK_b: S** is the supernatant fraction (0.46 µg) of higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex; **X-K_cK_b/E2-X-K_cK_b: P1, P2, P3** are pellet fraction of higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex loaded at three levels (0.58 µg, 0.29 µg, 0.14 µg); **E2-X-K_cK_b: S** is the supernatant fraction (0.18 µg) of control E2-X-K_cK_b subcomplex; **E2-X-K_cK_b: P** is the pellet fraction (0.21 µg) of control E2-X-K_cK_b subcomplex; **X-K_cK_b: S** is the supernatant fraction (0.44 µg) of control X-K_cK_b fraction; **X-K_cK_b: P** is the pellet fraction (0.02 µg) of control X-K_cK_b fraction; **X-K_cK_b** is the X-K_cK_b fraction loaded at two levels (1.0 µg, 0.5 µg); **PDC** is pyruvate dehydrogenase complex (1.0 µg).

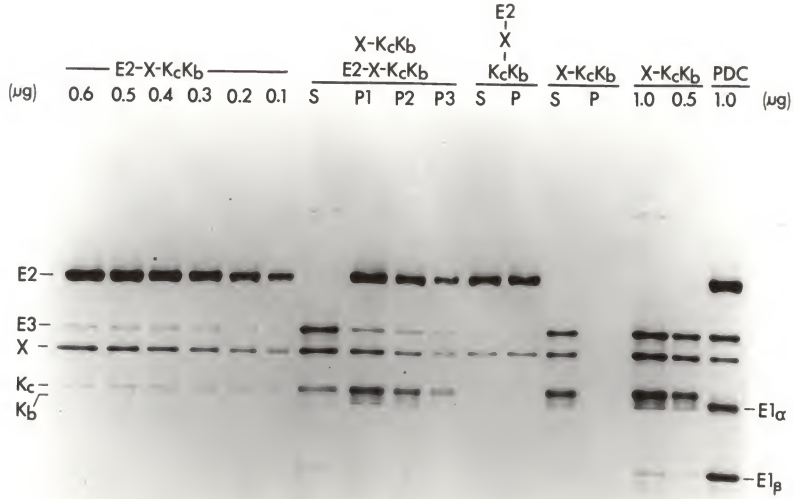
E2
X
X-KcKb
E2-X-KcKb
KcKb
X-KcKb
X-KcKb
PDC
E2-X-KcKb
(μg)
0.6 0.5 0.4 0.3 0.2 0.1
S P1 P2 P3
S P
S P
1.0 0.5
1.0
(μg)
E2
E3
X
Kc
Kb
E1α
E1β

Figure 1-4. SDS-PAGE of PDH kinase binding with E2-X_I subcomplex and $E2^*$ subcomplex. 10 µg X-K_cK_b fractions were incubated with 20 µg E2-X_I subcomplex and 20 µg $E2^*$ subcomplex repectively, binding experiments were performed essentilly the same as described before; First four lanes from left are E2-X-K_cK_b subcomplex standards (0.4 µg-0.1 µg); **X-K_cK_b/E2-X_I: S** is the supernatant fraction (0.36 µg) of X-K_cK_b fraction/E2-X_I subcomplex; **X-K_cK_b/E2-X_I: P1, P2, P3** are three levels of the pellet fraction (0.9 µg, 0.45 µg, 0.2 µg) of X-K_cK_b fraction/E2-X_I subcomplex; **X-K_cK_b/$E2^*$: S** is the supernatant fraction (0.48 µg) of X-K_cK_b fraction/$E2^*$ subcomplex; **X-K_cK_b/$E2^*$: P1, P2, P3** are three levels of the pellet fraction (0.7 µg, 0.35 µg, 0.2 µg) of X-K_cK_b fraction/$E2^*$ subcomplex; **X-K_cK_b: S** is the supernatant fraction (0.24 µg) of control E2-X_I subcomplex; **X-K_cK_b: P** is the pellet fraction (0.45 µg) of control E2-X_I subcomplex; **$E2^*$: S** is the supernatant fraction (0.48 µg) of control $E2^*$ subcomplex; **$E2^*$: P** is the pellet fraction (0.33 µg) of control E2* subcomplex; **X-K_cK_b** is X-K_cK_b fraction (1.0 µg); **PDC** is pyruvate dehydrogenase complex (1.0 µg).

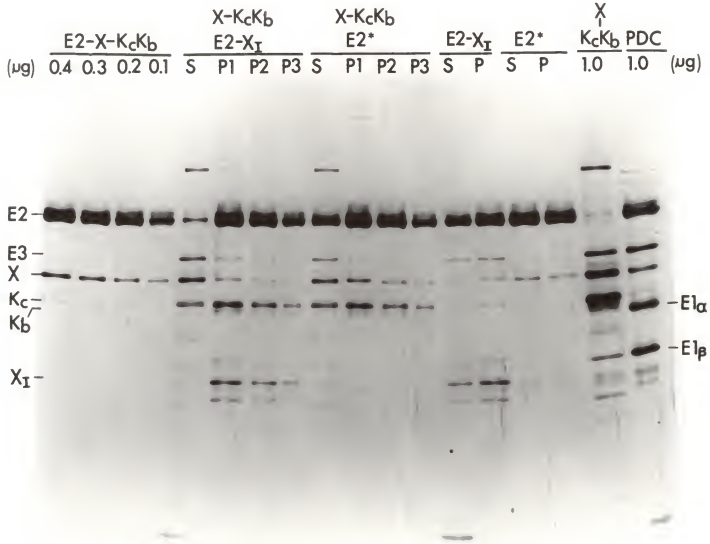
E2-X-KcKb
X-KcKb
E2-XI
X-KcKb
E2*
E2-XI
E2*
X
I
KcKb
PDC
(μg) 0.4 0.3 0.2 0.1 S P1 P2 P3 S P1 P2 P3 S P S P 1.0 1.0 (μg)
E2
E3
X
Kc
Kb
XI
E1α
E1β

Table 1-I. PDH kinase ATP binding capacity, specific activity and transacetylation activity in various kinase-containing fractions.

Fraction	[^{14}C]ATP Bound[a] (nmole/mg)	Specific Activity[b] (nmole/min/mg)	E2 Activity[c] (nmole/min/mg)
E2-X-K_cK_b	0.50	9.1	4.4
X-K_cK_b	3.28	-	-
Lower X-K_cK_b/E2-X-K_cK_b			
Supernatant	0.51	16.5 (17.6)[d]	-
Pellet	1.35	54.3 (49.7)[d]	2.6
Higher X-K_cK_b/E2-X-K_cK_b			
Supernatant	1.53	39.1 (26.5)[d]	-
Pellet	2.84	84.9 (56.9)[d]	2.9

[a]PDH kinase ATP binding was measured with 180 μM [^{14}C]ATP under the conditions described in methods. 12.2 μg E2-X-K_cK_b subcomplex, 4.0 μg X-K_cK_b fraction, 5.1 μg supernatant and 7.8 μg pellet fraction of lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex, 6.9 μg supernatant and 8.7 μg pellet of higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex was used respectively with each assay.

[b]PDH kinase activity were corrected for low kinase activity in E1 component and E2-X subcomplex. 22 μg E1 component, 5 μg E2-X subcomplex were used in the assay. Kinase-containing fractions added were as follows: 0.51 μg supernatant or 1.2 μg pellet fraction of lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex, 0.46 μg supernatant or 1.2 μg pellet fraction of higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex, for kinase in E2-X-K_cK_b subcomplex, 5 μg E2-X-K_cK_b subcomplex was assayed with 22 μg E1 component only.

[c]E2 activity was carried out at the presence of 0.5 mM [1-^{14}C]acetyl-CoA and 0.5 mM DHL. Amount of each E2 source added was as follows: 6.1 μg E2-X-K_cK_b subcomplex, 3.9 μg pellet fraction of lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex, 4.1 μg pellet fraction of higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex.

[d]Numbers in the parenthesis represent the kinase activity assayed without addition of E2-X subcomplex.

Table 1-II. Number of PDH kinase binding sites on E2 determined from lower X-K_cK_b fraction/E2-X-K_cK_b subcomplex and higher X-K_cK_b fraction/E2-X-K_cK_b subcomplex binding experiments.

Fraction	ATP binding site	Max. possible ATP binding site
	E2 core	E2 core
Lower X-K_cK_b/E2-X-K_cK_b Pellet Fraction	9	10
higher X-K_cK_b/E2-X-K_cK_b Pellet Fraction	16	27

Calculation is based on the ATP binding site per E2 catalytic unit.

Calculation of kinase binding sites of higher X-K_cK_b/E2-X-K_cK_b pellet fraction

1) E2-X-K_cK_b subcomplex:
   (0.5 nmole ATP/mg)/(4.4 E2 unit/mg) = 0.11 nmole ATP/E2 unit
2) Pellet fraction of higher X-K_cK_b/E2-X-K_cK_b:
   (2.84 nmole ATP/mg)/(2.9 E2 unit/mg) = 0.98 nmole ATP/E2 unit
3) Kinase binding percentage increase:
   (0.98 nmole ATP/E2 unit)/(0.11 nmole ATP/E2 unit) x 100%
   = 890%
4) Assume molecular weight of E2-X-K_cK_b subcomplex is 3.7×10^6, the kinase molecules present in the subcomplex is:
   0.5 nmole ATP/mg E2-X-K_cK_b x 3.7×10^6 mg/mmole
   = 1.85 mole ATP/mole E2 core
5) Number of kinase molecules bound:
   1.85 x 890% = 16 mole ATP/mole E2 core

Maximum possible kinase binding sites calculation

1) kinase units in X-K_cK_b fraction:
   3.28 nmole ATP/mg x 0.06 mg = 0.1968 nmole ATP
2) Kinase units in E2-X-K_cK_b subcomplex:
   0.5 nmole ATP/mg x 0.03 mg = 0.015 nmole ATP
3) Total kinase units:
   0.1968 + 0.015 = 0.2118 nmole ATP
4) Total E2 catalytic units:
   4.4 E2 unit/mg x 0.03 mg = 0.132 E2 units
5) The ratio of kinase units:E2 units:
   0.2118 nmole ATP/0.132 E2 unit = 1.60 nmole ATP/E2 unit
6) Maximum possible kinase binding percentage increase:
   (1.60 nmole ATP/E2 unit)/(0.11 nmole ATP/E2 unit) x 100%
   = 1458%
7) Mamimum possible kinase binding sites:
   1.85 x 1458% = 27 mole ATP/mole E2

Chapter 2

Study of Reductive Acetylation Reaction

INTRODUCTION

Pyruvate dehydrogenase complex catalyzes a series of coordinated reactions (figure 2-1):

$CH_3COCOOH + [TPP]-E1 \longrightarrow$

$$[CH_3C(OH)=TPP]-E1 + CO_2 \qquad (1)$$

$[CH_3C(OH)=TPP]-E1 + [lipS_2]-E2 \rightleftharpoons$

$$[CH_3CO-S-lipSH]-E2 + [TPP]-E1 \qquad (2)$$

$[CH_3CO-S-lipSH]-E2 + CoA-SH \rightleftharpoons$

$$[lip(SH)_2]-E2 + CH_3CO-S-CoA \qquad (3)$$

$[lip(SH)_2]-E2 + [FAD]-E3 \rightleftharpoons$

$$[lipS_2]-E2 + \text{reduced } [FAD]-E3 \qquad (4)$$

reduced $[FAD]-E3 + NAD^+ \rightleftharpoons$

$$[FAD]-E3 + NADH + H^+ \qquad (5)$$

The pyruvate dehydrogenase (E1) component catalyzes reactions 1 and 2. The dihydrolipoyl transacetylase (E2) component catalyzes reaction 3. Reaction 4 and 5 are catalyzed by the dihydrolipoyl dehydrogenase (E3) component.

A side reaction which is catalyzed by the E1 component is stimulated by acetaldehyde to make acetoin:

$[CH_3C(OH)=TPP]-E1 + CH_3CHO \rightleftharpoons$

$$CH_3CH(OH)COCH_3 + [TPP]-E1 \qquad (6)$$

All the reactions are reversible except the decarboxylation of pyruvate in reaction 1.

PDH isolated from bovine kidney and heart PDC has an $\alpha_2\beta_2$

tetramer structure (1). The approximate molecular weights of the α subunit and β subunit are 41,000 and 36,000 respectively. Pyruvate dehydrogenase kinase catalyzes the phosphorylation and inactivation of pyruvate dehydrogenase component (3,6). Phosphorylation covalently modifies the α subunit of PDH (1), but the mechanism by which this leads to inhibition of the overall reaction is not established. PDH phosphatase, another regulatory enzyme loosely associated with PDC, dephosphorylates the inactive form of PDH and converts it into the active form (4).

The E1 catalyzed decarboxylation reaction requires thiamin pyrophosphate (TPP) binding to the E1 component. Phosphorylation of the E1 α subunit increases its K_d for TPP about 2-fold (from 7 μM to 15 μM) (8). Reaction of a lysine residue of the E1 α subunit with pyridoxal 5'-phosphate inhibited the PDH activity and caused a 50-fold increase in K_d for thiamin-PP (7). Thus it is likely that TPP is bound primarily or exclusively by the E1 α subunit.

Phosphorylation of the PDH component markedly inhibited the PDC overall reaction with pyruvate as substrate, but when HETPP was used, there was no effect on the much slower rate of the reductive acetylation reaction (reaction 2) (6). The decarboxylation of pyruvate (reaction 1) was inhibited due to the phosphorylation of the α subunit. Based on this observation, it was proposed that the α subunit, which bears the phosphorylation sites, catalyzes the decarboxylation reaction, and the β subunit catalyzes the reductive acetylation reaction. However, the role

of each subunit has not been established yet, apparently due to difficulties in obtaining native α and β subunits of E1 component.

Interest in testing the potential catalytic function of the E1 β subunit was stimulated by the finding that the E1 α subunit could be selectively cleaved by trypsin and that the residual E1 β subunit retained the capacity to bind to E2 subunits (reference (18) in Chapter 1).

The PDC can slowly catalyze the reverse of the reductive acetylation reaction. This 'partial back reaction' was studied by Walsh *et al.* (8). This is a TPP dependent and acetyl-CoA dependent reaction. Walsh *et al.* measured the rate of acetyl-CoA-dependent oxidation of NADH in the presence of acetaldehyde which would react with the hydroxyethylidene thiamin pyrophosphate (HE=TPP) to yield acetoin. A basal oxidation of NADH was present and the correction for this background was significant. An assay which directly measures the yield of acetoin, a by-product formed by HE=TPP with acetaldehyde of reaction 6 following the reverse of the reductive acetylation reaction catalyzed by E1 (reverse of reaction 2) was developed in this study to attempt to gain insight into the function of E1 β subunit and the effect of phosphorylation on the PDC catalyzed reverse reaction.

MATERIALS AND METHODS

Materials: PDC was isolated from bovine kidney and separated into its component enzymes as described by Linn *et al.* (5). The E1 β subunit-rich fraction was obtained by trypsin treatment and ammonium sulfate fractionation. [1-^{14}C]acetyl-CoA was purchased from NEN Du Pont Company. Dowex 1-X8 anion exchange resin (20-50 mesh, chloride form) was from Bio-Rad. Organic acid analysis column used to separate acetoin was from Bio-Rad. All other chemicals used were the highest grade available.

Inactivation of pyruvate dehydrogenase complex: 0.1 ml PDC (17.9 mg/ml) was incubated with 0.6 mM ATP and 3 mM $MgCl_2$ at 30°C for 10 min and then on ice for 15 minutes. A G-50 sephedex column was used to remove the excess ATP.

Reverse reductive acetylation assay: The assay was conducted in a reaction mixture containing 0.5 mM TPP, 1 mM NADH, 0.4 mM NAD^+, 1 mM $MgCl_2$, 0.5 mM EDTA (ethylenediamine tetraacetic acid), 5 mM acetaldehyde, 50 mM K_xPO_4, pH 7.5 and 0.2 mM [1-^{14}C]acetyl-CoA (20,000 cpm/nmole) in a total volume of 0.1 ml. Purified PDC or resolved PDC subcomplexes were added at indicated levels. The reaction was initiated by adding [1-^{14}C]acetyl-CoA. The mixture was incubated in water bath at 30°C. At designated time intervals, a portion of the reaction mixture was withdrawn and the reaction was terminated by addition of perchloric acid (PCA) to a final PCA concentration of 4%. After incubation on ice , the

protein was pelleted by centrifugation in a minifuge (8000xg). This was repeated until no visible precipitate was present in the supernatant. The PCA in the supernatant was removed by neutralizing with KOH and potassium salt was pelleted by centrifugation in a minifuge several times until all the visible salt was removed. This solution, containing both radioactively labeled acetyl-CoA and acetoin, was loaded on anion exchange column and equilibrated for 2-3 min for the absorption of acetyl-CoA onto the anion exchange resin. The sample was collected by centrifugation from the column in a clinical centrifuge. To reduce the non-specific acetoin binding on the resin, the column was rinsed with 50 mM non-radioactive ('cold') acetoin solution before the fraction containing the radioactive acetoin was applied. The used resin was regenerated by rinsing the column with large volume of concentrated HCl to elute bound acetyl-CoA. Acetyl-CoA sticks to the radioactivity detector cell and may cause problems in the radioactivity detection system (see below).

Acetoin detection by high performance liquid chromatography (HPLC): The sample was analyzed using an isocratic HPLC system. An organic acid column for separating small organic molecules was employed. Filtered and degassed 0.01 N H_2SO_4 was used as solvent and the flow rate was set at 0.8 ml/min. The radioactive acetoin was detected by Packard Trace 7140 radioactivity flow monitor. The acetoin had a characteristic retention time of about 15 minutes (Figure 2-3).

RESULTS

Rate of the reverse reaction.

The reverse of the reductive acetylation reaction was carried out with high NADH:NAD^+ ratio (2.5), high concentration TPP (0.5 mM) and acetaldehyde (5 mM) to drive it backward to produce acetoin. The time course of this reaction was shown in figure 2-4. The rate of acetoin production was 0.11 µmole/mg PDC/min.

The function of E1 β subunit.

The role of E1 β subunit was studied by this reverse reaction. The trypsin treatment of E1 cleaved E1 α subunit and generated a fraction that was rich in E1 β subunit. The E1 β subunit was found to bind E2 subunit indicating that β subunit retained native structure. E1 β subunit was reconstituted with E2 and E3 in reaction mixture and incubated at 30°C for 2 hours. There was no observable acetoin produced; whereas when intact E1 component was reconstituted with E2 and E3, the full activity was restored. This indicated that E1 β subunit alone was not able to catalyze the reaction.

The effect of PDC phosphorylation on the reverse reaction.

Two assays were conducted, one used 16 µg phosphorylated PDC, the other used 14.5 µg nonphosphorylated PDC as control. Both reactions were incubated at 30°C for 2 hours. Acetoin production was completely inhibited in the one done with phosphorylated PDC, while the nonphosphorylated PDC catalyzed the reaction.

DISCUSSION

Although it is well known that phosphorylation of pyruvate dehydrogenase (E1) component greatly inhibits the overall PDC catalyzed reaction, how and at which step it causes inhibition is still not fully characterized yet.

Walsh *et al.* (8) studied the 'partial back reaction' using NADH oxidation assay. This reaction gave a background of NADH oxidation due to the reduction of E2 lipoyl domain without being acetylated. The reaction rate was determined to be 0.055 μmole/min/mg. The rate is slower than the rate measured in this study (0.11 μmole/min/mg). Since the PDC they used was about one half of the specific activity used in this study, the reaction rates may be similar. In both studies acetaldehyde was used as an acceptor for HE=TPP formed to speed up the reaction. The reverse of the reductive acetylation reaction developed in this study used acetaldehyde to trap HE=TPP and form acetoin as a product, and by removing this product the back reaction was enhanced. This was achieved by using a reaction mixture containing high ratio of $NADH/NAD^+$, high TPP concentration to facilitate its binding to E1 ([TPP]-E1). The acetyl group from acetyl-CoA was transferred to [TPP]-E1 to form [HETPP]-E1, probably in the hydroxyethylidene form, in the presence of acetaldehyde and H^+, acetoin was formed.

Apparently contradictory results were obtained from the study of the reductive acetylation reaction by Roche *et al.* and Walsh *et al.* (6,8). This reaction is the rate-limiting step of the PDC catalyzed overall reaction (2). Roche *et al.* used HETPP as

substrate and studied the reductive acetylation reaction as a model reaction. This reaction is much slower than the PDC catalyzed reductive acetylation reaction. They demonstrated that phosphorylated PDC inhibited the overall reaction when pyruvate was the substrate, but when HETPP was added as substrate, the utilization of HETPP was not affected by phosphorylation using NAD^+ reduction assay. Reductive acetylation assay revealed that phosphorylation of E1 α subunit inhibited the pyruvate decarboxylation reaction, but not reductive acetylation reaction (6).

Randle *et al.* studied the effects of phosphorylation on PDC and they came to the conclusion that all reactions in which [HE=TPP]-E1 was formed were inhibited by phosphorylation of the enzyme and no reaction in which [HE=TPP]-E1 was not involved was affected (8). In the present study phosphorylation of PDC eliminated the acetoin production by reverse reaction completely. Thus the result seems to agree with their observation.

There are some differences between the forward and reverse reactions. In the reverse reaction [HE=TPP]-E1 reacts with acetaldehyde to form acetoin and TPP-E1, TPP-E1 can then be directly used in next round reaction (figure 2-2). When HETPP is used as substrate in the forward reaction, the rate limiting step is either deprotonation of [HETPP]-E1 to form [HE=TPP]-E1, or TPP dissociation from E1 component so that E1 subunit can be recycled to bind HETPP (figure 2-2).

However because these reactions involving HETPP are complex

and have multiple steps, it is still not clear how phosphorylation inhibits these reactions. The phosphorylation increases K_d of E1 for TPP about two-fold, however this increase seems inadequate to account for the large activity difference between the active and inactive forms of PDC.

Several possible explanations are considered based on the observations mentioned above. Firstly, although phosphorylation of the E1 α subunit does not affect the TPP binding greatly, it may alter the orientation of bound TPP. Secondly, phosphorylation may modify the decarboxylation active site on the E1 component, thus blocking the ability of TPP-E1 to catalyze the decarboxylation of reaction 1, or alter the reductive acetylation active site, so inhibiting the acetyl group transfer of reaction 2. Another possibility is that phosphorylation blocks the HE=TPP translocation between the two active sites on E1 component. Although HE=TPP is made at the reductive acetylation active site on E1 component, phosphorylation inhibits the translocation of HE=TPP to decarboxylation active site where it reacts with acetaldehyde, therefore acetoin can not be made. Also the reductive acetylation reaction requires the attachment of the E1 component to a binding region on the E2 subunit to bring enzyme components to the best proximity to catalyze the reaction. The reductive acetylation reaction requires structure of the lipoyl domain of the E2 subunit. Because phosphorylation of E1 component may cause improper positioning of E1 component on E2 subunits, it is likely that the lipoyl domain of E2 subunit is not able to

enter the reductive acetylation active site properly on E1 component and the transfer of acetyl group between E1 and E2 subunits can not be efficiently accomplished.

New evidence has suggested that an acetyl-TPP is formed as an intermediate from the oxidation of HE=TPP, with the concomitant reduction of the lipoyl moiety of the E2 subunit. Phosphorylation may also block the formation of acetyl-TPP from HE=TPP or the subsequent transfer of the acetyl group to the reduced lipoyl moiety. The mechanism of PDC activity inhibition by phosphorylation still remains to be further studied.

Stepp *et al.* (7) found that PDC could be inactivated by pyridoxal 5'-phosphate, and the reaction site of pyridoxal 5'-phosphate was determined to be a lysine residue of the E1 α subunit (7). Also, as mentioned above, phosphorylation of E1 component markly inhibited the reaction 1 but did not inhibit the reaction 2 (6). Since the α subunit of the E1 component undergoes phosphorylation, it was assumed that E1 α subunit was the functional subunit to catalyze the pyruvate decarboxylation reaction (reaction 1) and E1 β subunit was the one to catalyze the reductive acetylation reaction (reaction 2) based on the above observations.

To attempt to establish E1 subunit catalytic functions, E1 β fraction was studied using the reverse of the reductive acetylation reaction. The failure of E1 β subunit to restore reconstitution activity with E2 and E3 components indicated that E1 β alone was not able to catalyze reaction 2. Since E1 α

subunit is not available to examine the reconstitution activity, the conclusion can not be made. But it is most likely that active site resides between α and β subunits of E1 and requires both subunits to function.

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Figure 2-1. Coordinated reactions catalyzed by pyruvate dehydrogenase complex.

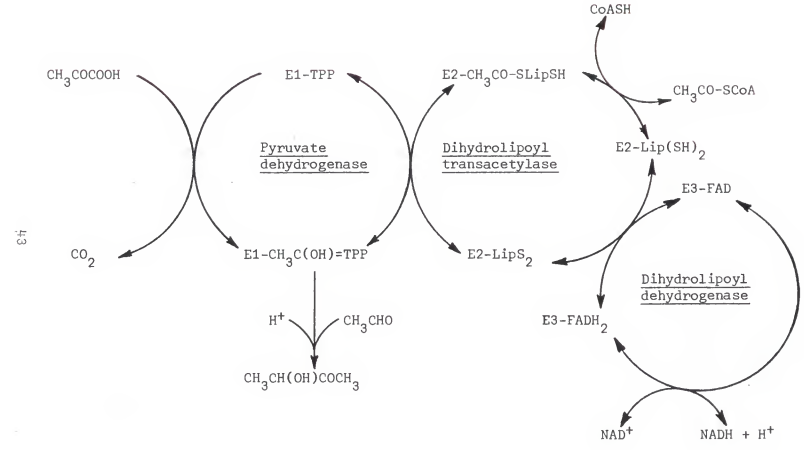
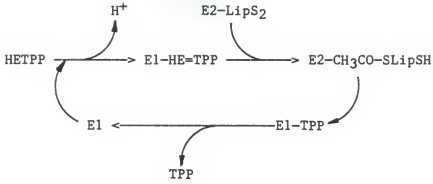
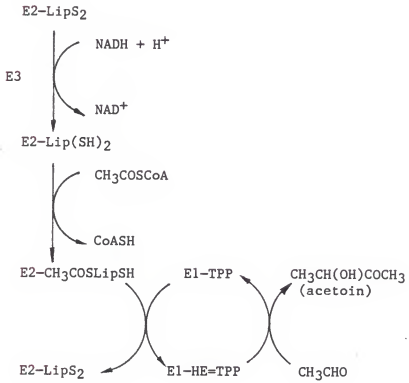

CoASH
CH3COCOOH
E1-TPP
E2-CH3CO-SLipSH
CH3CO-SCoA
Pyruvate dehydrogenase
Dihydrolipoyl transacetylase
E2-Lip(SH)2
E3-FAD
CO2
E1-CH3C(OH)=TPP
E2-LipS2
Dihydrolipoyl dehydrogenase
H+
CH3CHO
E3-FADH2
CH3CH(OH)COCH3
NAD+
NADH + H+

Figure 2-2. Forward and reverse reaction steps involving hydroxyethyl thiamin pyrophosphate (HETPP).



Forward reaction

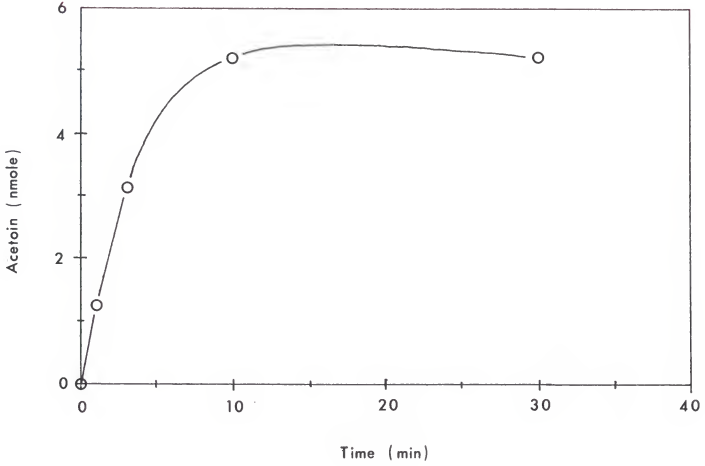


Reverse reaction

Figure 2-3. HPLC profile of acetoin.

1000
Injection
0
20
Time (min)
11.50
13.75
14.75
1000

Figure 2-4. Time course of acetoin production by reverse reductive acetylation reaction. Data represents nmole of acetoin produced in the assay mixture of 0.1 ml (refer methods for details in the text).


6
4
2
0
Acetoin (nmole)
0
10
20
30
40
Time (min)

Chapter 3

Effects of Extrinsic Factors on Pyruvate Dehydrogenase Complex

INTRODUCTION

Since pyruvate dehydrogenase complex catalyzes the reactions that serve both bioenergetic and biosynthetic roles, the regulation of this enzyme complex is of central importance to the energy balance and fuel economy of animal cells. The stimulation of PDC activity is one of the major sites of insulin regulation. Insulin was the first peptide hormone isolated and sequenced and the three dimensional structure elucidated. While many hormones which bind to cell surface receptors activate a second messenger generation system, it still remains largely unknown how insulin exerts the diverse effects on the intracellular regulation and function after it binds to the receptor on the plasma membrane. Over the past 20 years, a large number of attempts have been made to clarify the biological pathways involved in insulin signal transduction (12).

Some investigations have focused on free intramitochondrial Ca^{2+}, since it has been found that Ca^{2+} is the second messenger for several other hormones (α-adrenergic agonists, vasopressin, angiotensin *etc.*). However Denton's laboratory has presented evidence that the PDC activation by insulin does not involve an increase in free intramitochondrial Ca^{2+} concentration (4). Some other proposed mechanisms have also been proved not to be

primarily responsible for mediating the action of insulin. Since late 1970's and early 1980's, researchers have concentrated on looking for an insulin-dependent second messenger. After Jarett's laboratory presented evidence for a mediator of insulin effects on PDC (5,14), several other laboratories also presented the results supporting such modulator(s) (7,13). The mediator(s) found has been reported to be capable of regulating the activities of several insulin-sensitive enzymes such as PDC, cAMP phosphodiesterase (PDE), adenylate cyclase, etc. (5,8,9,10,13,14). The apparent molecular weight of the putative mediator(s) was determined to be about 1000 by gel permeation chromatography (8). The properties of the putative mediator(s) include net negative charge, stabililty to acid, and inactivation by chemical modification (12). Further studies support an inositol phosphate glycan structure based on the chemical properties (9,10). It is suggested that such mediators originated from the plasma membrane and might be released into the cytoplasm upon insulin binding to the receptor (8,9,10). The precursor(s) of the mediator(s) is thought to be anchored in the plasma membrane through a phosphodiester linkage between diacylglycerol and inositol (12). The generation of these substances from membranes by insulin can be reproduced by addition of a phosphatidyl inositol-specific phospholipase C (PIPLC) which acts on the linkage and releases the soluble glycan structure (9). This suggests that insulin may activate an intrinsic phospholipase C upon its binding to the receptor which in turn selectively hydrolyzes

phosphatidylinositol, releasing certain inositol phosphate glycan structures. In fact, an enzyme with these properties was found in the rat liver membrane fraction purified by Fox *et al.* (2). Recently, Saltiel *et al.* (11) demonstrated that the addition of IPG and PIPLC into adipocytes caused an increase in lipogenesis and glucose oxidation. These results suggest that IPG added or released from the adipocytes might serve as a second messenger.

Since PDC can be activated by insulin and this activation has been suggested to be achieved by either inhibition of kinase or activation of phosphatase or both (1), several studies on the insulin mediators have been conducted on PDC system. Seals and Jarret (14) showed that addition of insulin to a mixture of plasma membrane and mitochondria resulted in a decrease in the phosphorylation of the E1 α subunit (14). Jarret later provided the evidence that the activation of PDC by insulin-generated mediator was due to the stimulation of PDH phosphatase (3).

Based on the present knowledge about insulin mediator, we have attempted to confirm observations on PDC that have been observed in other laboratories. The major goal of this study is to determine whether the PIPLC treated membrane fraction which presumably contains the potential messenger for mediating insulin induced activation of PDC, acts directly on the purified phosphatase or requires additional components present in responsive mitochondria. We also investigated the effects of extrinsic factors on the PDC activity in adipocytes.

MATERIALS AND METHODS

Materials: Male Sprague Dawley rats (100-150 g) were from Sasco. Highly purified PDC (15-18 μmole/mg/min) was used. Phosphatase was partially purified using DEAE-Sepharose column. Collagenase (type II) was purchased from SIGMA, [1-^{14}C]pyruvate (sodium salt) was from New England Nuclear (NEN) Du Pont Company. myo-inositol 2-monophosphate (IP) was purchased from SIGMA. Isolated insulin mediator fractions and phosphatidylinositol-specific phospholipase C (PIPLC) were generously provided by Dr. Ruth Welti from Division of Biology. All other chemicals used were highest grade available.

Brief mediator preparation steps: Mediator fractions were prepared in Dr. Ruth Welti's laboratory following the procedure described by Saltiel *et al.* (9). A particular fraction of rat liver membrane was purified by centrifugation. This fraction was lyophilized and resuspended in acetate buffer, pH 4.5, then treated with PIPLC. After incubation, samples were acidified to pH 4.0, lyophilized and redissolved in deionized water, pH 7.0-7.5. This was the crude mediator fraction. Further purification steps were performed by DEAE-cellulose column, C_{18} reverse-phase resin, Dowex AG 50W-X-4 anion-exchange resin, QAE-Sephadex column and Bio-Gel P-2 column. The fractions were lyophilized and resuspended in 50 mM formic acid.

Phosphatase assay: Typically the assay was conducted in a

mixture containing 30 mM MOPS-K^+, pH 7.5, 0.8 mM EDTA, 2 mM $MgCl_2$, 2.2 mM $CaCl_2$ and 2 mM EGTA (ethyleneglycol-bis-tetraacetic acid). Phosphorylated PDC, phosphatase and insulin mediator fractions were added at the indicated amounts. The reaction mixture was preincubated without the presence of Mg^{2+} and Ca^{2+} at 30°C for 1 min. The reaction was initiated by adding Mg^{2+} and Ca^{2+} and further incubated for 2 min. Total volume of each assay was 20 µl. A control was done without Mg^{2+} and Ca^{2+}. 10 µl was withdrawn and added into a temperature equilibrated cuvette at 30°C containing 2 mM $MgCl_2$, 2 mM NAD^+, 0.2 mM thiamin-PP, 50 mM K_xPO_4, pH 8.0, 62.5 µM CoA, 1.32 mM cystein-HCl and 2 mM pyruvate. The absorbance change rate at 340 nm was monitored.

Rat liver mitochondria isolation: The preparation of rat liver mitochondria essentially followed the procedure described by Saltiel *et al.* (7). All the steps were conducted at 4°C. Rat livers were rinsed three times in 20 mM K_xPO_4, pH 7.4, then chopped into small pieces, homogenized in 10 volumes of buffer A pH 7.2 (0.21 M mannitol, 75 mM sucrose, 1 mM tris, and 0.1 mM EDTA). They were centrifuged at 500xg for 10 min, and the pellet was discarded. The supernatant was centrifuged at 9000xg for 10 min, and the pellet was resuspended in equal volume of buffer A. The above two centrifugation steps were repeated once. The pellet was swollen to open the inner mitochondria membrane to enhance the enzyme sensitivity by adding 10 volumes of buffer B pH 7.2 (20 mM K_xPO_4 with 0.02 % BSA w/v) gradually over 45 min, while the pellet

was gently agitated frequently. Then 2 µg/ml oligomycin was added to the mitochondria. To inactivate the PDC in mitochondria, the suspension was incubated with 0.2 mM ATP at the presence of 50 µM $MgCl_2$ and $CaCl_2$ at 30°C for 10 min, then centrifuged at 30,000xg for 30 min to remove the excess ATP. The pellet was resuspended in buffer B and centrifuged again at 1900xg for 15 min. The final pellet was resuspended in buffer B, aliquoted and stored at -70°C. The protein concentration was measured by Biuret method.

Rat adipocyte isolation: The adipocytes were isolated by the procedure of Rodbell (6) with modification. Rat epidermal fat pads were rinsed in 0.85% NaCl and then put in 3 volumes of Krebs-Ringer buffer with 3% (w/v) BSA and 5 mM glucose, chopped into fine pieces. Collagenase (5 mg/g fat pad) was added into the mixture. The tube was flushed with 5% CO_2, 95% O_2 gas mixture and digested at 37°C in the water bath for one hour with shaking. The suspension was then passed through a nylon mesh to collect the adipocytes. After a brief spin (15 s) in the clinical centrifuge the cells floated to the top and the solution was carefully removed from the bottom. Cells were washed 3 times with 10 ml warm Krebs-Ringer buffer and finally suspended in an appropriate amount of the same buffer. 2 ml aliquots of cells were distributed to flasks and gassed with 5% CO_2, 95% O_2 mixture. The flasks were sealed and incubated at 37°C in a shaking water bath for 40 min to allow the insulin receptors to recover. Further incubation was conducted at same the temperature for 1 h with

extrinsic factors. The cells were broken using a Tekmar homogenizer and then transferred into eppendorf tube. The mitochondria were centrifuged three min by minifuge (8000xg). The pellets were resuspended in 0.8 ml stop solution (20 mM K_xPO_4, pH 7.5, 20 mM EDTA, 50 mM NaF, 4 % (v/v) Triton X-100 and 10 mM dichloroacetic acid (DCA)) and stored at -70°C.

Pyruvate dehydrogenase assay: For rat liver mitochondria PDH assay, the frozen sample was thawed and typically 0.5-0.6 mg protein was added to each assay mixture containing 1 mM DTT, 0.05 mM $MgCl_2$, 0.05 mM $CaCl_2$, 20 mM K_xPO_4, pH 7.5 in final volume of 1 ml. The mediator fractions were added to the mixture at indicated levels and incubated at 37°C for 10 min. The full activation extent of PDC was determined by incubating mitochondria with 20 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Then 0.2 ml of incubation mixture was added into the temperature equilibrated (30°C) tube containing 2.0 mM CoA, 10 mM DTT, 2.5 mM NAD^+, 0.25 mM TPP, 5 mg/ml BSA, 50 mM K_xPO_4, pH 7.5, 2 mM $MgCl_2$ and 4% Triton X-100. For the adipocyte mitochondria assay, the samples were thawed and 0.2 ml aliquots were added into the same solution. The mitochondria were preincubated for 1 min and the reaction was initiated by adding 220 nM [1-^{14}C]pyruvate (5000 cpm/nmole). The samples were sealed with a rubber cap with a plastic well attached to it. After 2 min the samples were placed in isopropanol-dry ice to stop the reaction. 0.2 ml β-phenylethylamine was injected on the filter paper in the plastic well. Radiolabeled CO_2 was released from the

solution by shaking at 37°C in the water bath for 30 min and absorbed by the filter paper, the plastic well was cut into the scintillation fluid and counted. All the samples were done in triplicate.

RESULTS AND DISCUSSION

The effects of mediator fractions on phosphatase activity.

Non-PIPLC treated and PIPLC treated crude fractions (see methods) which were prepared in Dr. Welti's laboratory were tested on PDC phosphatase assay. Low Mg^{2+} concentration (K_m) was used in the phosphatase assay. Initially 1 µl PIPLC treated fraction activated phosphatase activity by 60%, whereas the same amount without PIPLC treatment inhibited the activity by 34% compared with control (Table 3-I). When assayed with fresh phosphatase even further activation up to 190% was observed, and the fraction without treatment of PIPLC showed 65% inhibition (Table 3-I). However it seemed that the mediators were not very stable; the extent of activation ranged from 80% to 190% of the control value. The same PIPLC treated fraction was used at different concentrations to investigate the effect on phosphatase activation. When 0.5 µl and 1.0 µl of the PIPLC treated fraction were tested, 39% and 50% activations were observed respectively. However when the amount increased to 2.0 µl, it inhibited the activity by 61% (Table 3-I). As a biphasic tendency was observed, further characterization of both PIPLC treated fraction as well as untreated fraction was performed (Figure 3-1). PIPLC treated fraction at different concentrations exhibited a biphasic response. This agrees with the Saltiel's observation that the activation increases in proportion to the concentration of the mediator fraction and then declined at high concentrations (7). Saltiel suggested that this resulted from the existence of two

opposing substances. They provided the evidence by separating them by ethanol extraction. The ethanol-extracted residue (aqueous phase) had a consistent dose dependent stimulatory effect, whereas the ethanol-extractable (organic phase) gave a dose dependent decrease in the PDH activity. It seems that this could be a plausible explanation for the observation obtained from PIPLC treated fraction. But the fraction without the treatment of PIPLC also exhibited a biphasic pattern that was similar to PIPLC treated fraction, with the highest activation (86%) being shifted to the higher concentration (Figure 3-1). The reason why this happened is not known.

It has been determined that phosphatase activity requires millimolar Mg^{2+}, is increased by micromolar Ca^{2+}. The K_m for Mg^{2+} is 2-5 mM. In order to determine whether the observed activation by mediator fraction was K_m or V_{max} effect, titration of Mg^{2+} were done with and without the addition of PIPLC treated fraction on phosphatase assay (Figure 3-2). The extents of phosphatase activation by PIPLC treated fraction were large (45-66%) and only observed at very low Mg^{2+} concentrations (0.2-0.3 mM). At higher Mg^{2+} concentrations, a large phosphatase activation effect was not achieved. One obvious problem was that the effects were only seen at low (nonphysiological) Mg^{2+} concentrations. This may be attributed to the contamination of metal ions in the mediator fraction. However, over the entire range of Mg^{2+} tested, a consistently higher level of phosphatase activity was observed when assayed with PIPLC treated fraction. Furthermore, the

mediator fraction alone (i.e. in the absence of added Mg^{2+}, but in the presence of Ca^{2+}) failed to support any phosphatase activity (data not shown).

The effects of mediator fractions on PDC activity in mitochondria.

Both crude and further purified mediator fractions were tested with rat liver mitochondria. The mitochondria were prepared in a way so that the inner membrane was permeablized to enhance the enzyme sensitivity. The PDC in such prepared mitochondria was inactivated by ATP treatment and maintained the ability to be fully reactivated at the presence of Mg^{2+} and Ca^{2+}. Typically, a 15-fold increase in the activity of the PDC of ATP-treated mitochondria was obtained by high concentration of Mg^{2+} and Ca^{2+}. Fractions that were observed in Dr. Welti's lab to activate PDE were tested, but only limited effect (<10%) on PDC assay was observed. Considering its potential ability to be reactivated, the results were not encouraging. Other fractions were examined and hardly showed any sign of activation. It is possible that the mediator concentrations used in these assays were not appropriate for giving maximum activation. Due to the time consumed to prepare the mediator and the limited material available, the study was not carried out further.

The effects of extrinsic factors on PDC activity in adipocytes.

Saltiel *et al.* (11) recently demonstrated that incubating adipocytes with PIPLC increased the levels of lipogenesis and

glucose oxidation. Their results suggested that IPG which could mediate the insulin action was released from the plasma membrane. Also a mediator analogue IP has exhibited the ability to block the activation on lipogenesis and glucose oxidation by IPG. In this study the same system was employed to study the influences of insulin, IP and PIPLC on PDC activity (Table 3-1). Insulin activated the PDC activity with adipocyte cells increased but not as much as reported by others (e.g. 200% (1)). It is possible that during the collagenase digestion of fat tissues, the insulin receptors on the cell surface were partially impaired and not fully recovered during the recovery period. Two other experiments done with the addition of PIPLC yielded conflicting results (Table 3-II). PIPLC (1 µg/ml) alone and in combination with IP and insulin exhibited inhibitions compared to their respective controls. In a separate experiment, however, two concentrations of PIPLC (1 µg/ml, 2 µg/ml) were tested and they both showed an activation of near 30%.

The results from this study only represent some preliminary observations. It is still not well understood how insulin mediator regulates PDC activity. It seems that the putative insulin mediator is not very stable, since the same fraction used in the assay showed various activity at different times. Additionally the insulin mediator preparation procedure is time-consuming and the release of it can not be easily monitored during the isolation process. This can probably be improved by introducing radioactive label in the precursor of the mediator,

therefore its production is followed by radioactivity. Certainly, more investigation is needed in the future.

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Figure 3-1. The effect of fractions treated with and without PIPLC on the activity of phosphatase. The phosphatase assay was carried out with 20 ug phosphorylated PDC and 0.3 ug phosphatase. The reaction was initiated by adding 2 mM $MgCl_2$. Open circle represents the assays done with the PIPLC treated membrane fraction; Filled circle represents the assays conducted with the non-PIPLC treated membrane fraction (control). PIPLC treated and non-PIPLC treated membrane fractions used in this study were crude fractions (see methods in the text).

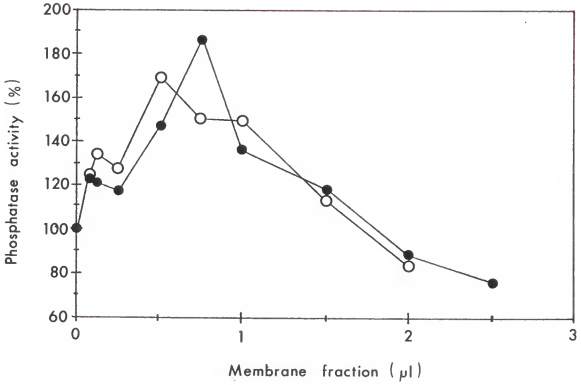
Phosphatase activity (%)
200
180
160
140
120
100
80
60
0
1
2
3
Membrane fraction (µl)

Figure 3-2. The activation of phosphatase activity at different Mg^{2+} concentration with and without PIPLC treated membrane fraction. The phosphatase activity was assayed with 20 µg phosphorylated PDC, 0.2 µg phosphatase at lower [Mg^{2+}] and 0.1 µg at higher [Mg^{2+}], 0.5 µl PIPLC treated membrane fraction was used (open circle); assays done without PIPLC treated membrane fraction (control; filled circle). Crude PIPLC treated membrane fraction was used (see methods in the text).

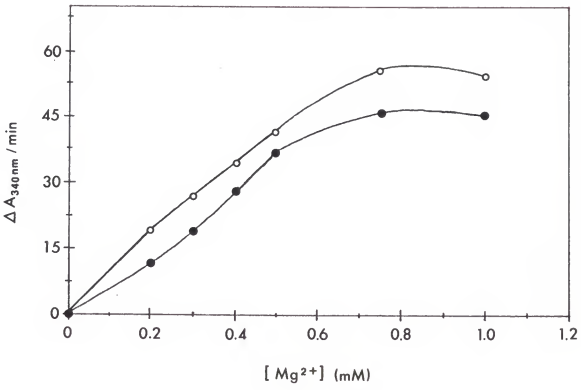
60
45
30
15
0
ΔA340nm / min
0
0.2
0.4
0.6
0.8
1.0
1.2
[Mg2+] (mM)

Table 3-I. Effects of PIPLC treated and non-PIPLC treated membrane fractions on phosphatase activity.

Fraction	Volume (µl)	Phosphatase Activity vs. control (%)
PIPLC treated fraction[a]	1.0	152
	1.0	163
Non-PIPLC treated fraction[a]	1.0	66
PIPLC treated fraction[b]	1.0	223
	1.0	290
	1.0	182
Non-PIPLC treated fraction[b]	1.0	35
PIPLC treated fraction[c]	0.5	139
	1.0	150
	2.0	39

[a]The phosphatase assayed with (0.3 µg) was 'old'.
[b]0.2 µg fresh phosphatase was used.
[c]0.3 µg phosphatase was assayed.

Table 3-II. Effects of extrinsic factors on PDC activity in adipocytes. 2 ml of adipocytes (3.8×10^5/ml) were incubated with each extrinsic factor or combination of them at 37°C for 1 hour, then mitochondria were isolated and assayed for PDC activity.

Extrinsic Factor	PDC Activity vs. control (%)
Insulin	
0.5 nM	145 (130)[c]
1.0 nM	148 (136)[c]
2.0 nM	143 (145)[c]
5.0 nM	136 (122)[c]
IP (1 mM)	127
PIPLC	
1 µg/ml	81 (133)[c]
3 µg/ml	(132)[c]
IP (1 mM) + Insulin (1 nM)	153
IP (1mM) + PIPLC (1 µg/ml)	138
Insulin (1 nM) + PIPLC (1 µg/ml)[a]	111
PIPLC (1 µg/ml) + Insulin (1 nM)[b]	112

[a]Insulin was preincubated with adipocytes for 3 minutes then PIPLC was added.
[b]PIPLC was preincubated with adipocytes for 3 minutes then insulin was added.
[c]Results obtained from a separate experiment, 2ml of adipocytes (9×10^5/ml) were used.

STUDIES ON THE MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX: PYRUVATE DEHYDROGENASE KINASE BINDING SITES, REDUCTIVE ACETYLATION REACTION AND EXTRINSIC CONTROL

by

LIN LI

B.Ed., Peking Institute of Physical Education, China, 1984

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirement for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group
Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1989

ABSTRACT

1. Through the resolution of purified bovine kidney pyruvate dehydrogenase complex (PDC), the following were prepared: dihydrolipoyl transacetylase-protein X-kinase ($E2\text{-}X\text{-}K_cK_b$) subcomplex, protein X-kinase ($X\text{-}K_cK_b$) fraction, E2 with inner domain of protein X ($E2\text{-}X_I$) subcomplex in which the outer domain of protein X was selectively removed and E2 oligomer with most protein X removed ($E2^*$). The fractions containing E2 and the $X\text{-}K_cK_b$ fraction were incubated and centrifuged. Sodium dodecyl sulfate gel electrophoresis of pellet fractions showed a significant increase in the binding of the catalytic subunit of the kinase (K_c) to E2 core, while the ratio of E2 to protein X stayed nearly the same. There was also increased binding of K_c to $E2\text{-}X_I$ and $E2^*$ subcomplexes, indicating that the binding of kinase to E2 is independent of protein X. These results provided evidence that 16 kinase molecules could be bound directly to the E2 core.

2. The reverse of the reductive acetylation reaction was studied through a coupled reaction involving the formation of acetoin through the reaction of the 2-(1-hydroxyethylidene)-thiamin pyrophosphate intermediate with acetaldehyde. The reaction rate was 0.11 μmole/mg/min. The phosphorylated PDC completely inhibited acetoin production. Following proteolytic cleavage of the E1 α subunit, the possibility that the β subunit of the E1 component upon reconstitution with E2 and E3 would catalyze the reductive acetylation reaction was evaluated. The

negative result indicates the E1 β subunit alone is not sufficient for catalyzing this reaction.

3. Phosphoinositol-specific phospholipase C (PIPLC) has been proposed to release from the plasma membrane a soluble inositolphosphate glycan that activates the PDC activity. The effects of PIPLC treated membrane fractions on the activation of PDC were investigated. The effects of the fractions were evaluated for their capacity to increase the activity of the purified pyruvate dehydrogenase phosphatase and to activate PDC in rat liver mitochondria and rat adipocytes. Some activation of phosphatase was observed with the phosphatase assay, but the extent of activation varied.